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Maternal exposure to a continuous 900-MHz electromagnetic field provokes neuronal loss and pathological changes in cerebellum of 32-day-old female rat offspring

Ersan Odacı [a,*], Hatice Hancı [a], Ayşe İkinci [a], Osman Fikret Sönmez [b], Ali Aslan [c], Arzu Şahin [c], Haydar Kaya [d], Serdar Çolakoğlu [e], Orhan Baş [f]

[a] Department of Histology and Embryology, Karadeniz Technical University, Faculty of Medicine, Trabzon, Turkey
[b] Department of Neurosurgery, Tepecik Education and Research Hospital, İzmir, Turkey
[c] Department of Physiology, Ordu University, Faculty of Medicine, Ordu, Turkey
[d] Department of Electrical and Electronic Engineering, Karadeniz Technical University, Faculty of Engineering, Trabzon, Turkey
[e] Department of Anatomy, Düzce University, Faculty of Medicine, Düzce, Turkey
[f] Department of Anatomy, Ordu University, Faculty of Medicine, Ordu, Turkey

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ABSTRACT

Large numbers of people are unknowingly exposed to electromagnetic fields (EMF) from wireless devices. Evidence exists for altered cerebellar development in association with prenatal exposure to EMF. However, insufficient information is still available regarding the effects of exposure to 900 megahertz (MHz) EMF during the prenatal period on subsequent postnatal cerebellar development. This study was planned to investigate the 32-day-old female rat pup cerebellum following exposure to 900 MHz EMF during the prenatal period using stereological and histopathological evaluation methods. Pregnant rats were divided into control, sham and EMF groups. Pregnant EMF group (PEMFG) rats were exposed to 900 MHz EMF for 1 h inside an EMF cage during days 13–21 of pregnancy. Pregnant sham group (PSG) rats were also placed inside the EMF cage during days 13–21 of pregnancy for 1 h, but were not exposed to any EMF. No procedure was performed on the pregnant control group (PCG) rats. Newborn control group (CG) rats were obtained from the PCG mothers, newborn sham group (SG) rats from the PSG and newborn EMF group (EMFG) rats from the PEMFG rats. The cerebellums of the newborn female rats were extracted on postnatal day 32. The number of Purkinje cells was estimated stereologically, and histopathological evaluations were also performed on cerebellar sections. Total Purkinje cell numbers calculated using stereological analysis were significantly lower in EMFG compared to CG ($p < 0.05$) and SG ($p < 0.05$). Additionally, some pathological changes such as pyknotic neurons with dark cytoplasm were observed in EMFG sections under light microscopy. In conclusion, our study results show that prenatal exposure to EMF affects the development of Purkinje cells in the female rat cerebellum and that the consequences of this pathological effect persist after the postnatal period.
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1. Introduction

According to the International Telecommunication Union, the number of mobile cellular subscriptions worldwide is almost the same as that of the total world population. The number of mobile cellular subscriptions at the end of 2104 was almost 7 billion, representing a penetration rate of 96% (ITU, 2014). This means that thousands of people are exposed to electromagnetic fields (EMF) of varying strengths from wireless devices (i.e. cell phones and cordless phones, cellular antennas and towers, and broadcast transmission towers), unwillingly and mostly unawares (Fragopoulou et al., 2010; Hardell and Sage, 2008).

The normal morphology and functioning of the neuronal system (NS) is connected with the prenatal and postnatal development of cerebellar cortex major neurons such as Purkinje cells. The developing NS is known to be vulnerable to potentially harmful factors such as chemical agents, environmental factors, drugs (Odaci et al., 2004; Ragbetli et al., 2007) and EMF exposure,

* Corresponding author.
E-mail addresses: eodaci@gmail.com, eodaci@yahoo.com (E. Odacı).

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especially during the prenatal term (Bas et al., 2009a, 2009b; Baş et al., 2013; İkinci et al., 2013; Odaci et al., 2008). The NS of an animal species being tested must be comparable to that of the equivalent stage of development in humans, regardless of whether it is tested during the fetal, perinatal or postnatal periods (Jacobson, 1991; Rodier, 1980). For example, the neonatal period of NS development in rats is equivalent to the third trimester of development in humans (Dobbing, 1970; Dobbing and Sands, 1973; Jacobson, 1991; Rodier, 1980). Therefore, a study of the effect of 900 MHz EMF during the neonatal period of rat NS development may be of some predictive value regarding the effects of EMF on the late human embryo. Additionally, examination of the effects of prenatal exposure to 900 MHz EMF on newborn rat cerebellar development may provide useful data for evaluating cerebellar malformations during pregnancy, since the available evidence for the developmental neurotoxicity of 900 MHz EMF on the cerebellum in the early postnatal period is insufficient.

Several previous studies have shown the effects of exposure to 900 MHz EMF during various gestational periods on the development and functions of the NS (Bas et al., 2009a, 2009b; Baş et al., 2013; İkinci et al., 2013; Odaci et al., 2008; Sonmez et al., 2010). The cerebellum has been shown to be particularly susceptible to exposure to 900 MHz EMF in these studies (Sonmez et al., 2010). Some evidence for altered female rat cerebellum development in association with prenatal exposure to 900 MHz EMF has recently been reported. Studies have suggested that the development of Purkinje cells in the cerebellar cortex may be affected following prenatal administration of EMF (Sonmez et al., 2010). However, insufficient data are still available regarding the impact of exposure to 900-MHz EMF during prenatal days 13–21 on later postnatal cerebellum development. This study was planned to investigate the 32-day-old female rat pup cerebellum following exposure to 900 MHz EMF during prenatal days 13–21 using stereological and histopathological evaluation methods.

2. Materials and methods

2.1. Animals, groups and experimental procedures

All rats used in this study were obtained from the Karadeniz Technical University Experimental Animals Surgical Research and Application Center. All animal experiments and procedures were compatible with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health, and the study was approved by the Karadeniz Technical University Animal Care and Ethics Committee, Turkey. At the beginning of the study, 40 rats were used to obtain female rat pups, 20 healthy Sprague Dawley female rats (exhibiting two regular cycles, 8–10 weeks old, weighing 180–250 g) and 20 Sprague Dawley male rats (8–10 weeks old, weighing 180–250 g). Rats were housed in the Karadeniz Technical University Experimental Animals Surgical Research and Application Center in standard plastic cages equipped with wood-shaving bedding at a temperature of 22 ± 1 °C under controlled lighting (12 h light/12 h dark cycle), and humidity (50 ± 10%). During the experiment, standard rat chow and tap water were provided ad libitum.

On the evening of the first day of the study, each female rat was placed in a separate cage containing a male rat and allowed to mate. Pregnancy was tested the following day using vaginal smears. Female rats were regarded as pregnant if sperm were observed in their smear specimens, and that day was recorded as day 0 of pregnancy. Nine rats were identified as pregnant, and these were randomly divided into three groups of three pregnant rats each. The first group was adopted as the pregnant control group (PCG), which was subjected to no procedures throughout pregnancy. The second group of mothers was adopted as the pregnant sham group (PSG). This group was placed inside a Plexiglas EMF cage at the same time every day between the 13th and 21st days of pregnancy for 1 h, during which time it was not exposed to any EMF. The third group of mothers was adopted as the pregnant EMF group (PEMFG). Rats in this group were placed inside the Plexiglas EMF cage and exposed to 900-MHz EMF for 1 h at the same time every day between the 13th and 21st days of pregnancy. A portable ultra-high-frequency oscillator (1218-BV, Lockable Oscillator, 900–2000 MHz, General Radio Company, Concord, Massachusetts, USA, Serial No. 1483) connected to an uninterrupted portable power source (1267-B Regulated Power Supply, General Radio Company, Concord, MA, USA, Serial No. 903) with an output power of approximately 300 mW and a frequency adjusted to 900 MHz was used to expose PEMFG to 900 MHz EMF. The oscillator was attached to a half-wave dipole antenna consisting of a 1 mm × 15 cm copper rod using a coaxial cable. The antenna was placed in the central area, approximately 11 cm inside the open surface of a Plexiglas cage produced specially for EMF investigation and measuring 30 cm × 42 cm × 50 cm. An insulating wooden rod was used in order to fix the antenna in the center of the cage. The rod was attached at an equal distance from the right and left and front and rear edges of the cage and was fixed in place between the right and left edges. This attached rod was determined as the central point and the copper rod used as an antenna was installed equidistant from right and left from that center (Fig. 1). The whole-body specific absorption rate (SAR) was calculated at 0.01 W/kg (RadHaz SAR Equivalence Calculator Version 1.0, © 2000 Richard Tell Associates, Inc., Mesquite, NV, USA).

Each pregnant rat was removed and placed in a separate cage before giving birth. After birth, pups remained in the same cages as their mothers and were not subjected to any procedures until postnatal day 32. On postnatal day 32 the female rat pups from each group were separated, and six female rat pups were selected at random from each group. The new groups established were classified as the control group (CG), sham group SG and EMF group (EMFG). The CG consisted of newborn female pups from the PCG rats, SG of newborn female pups from the PSG rats and EMFG of newborn female pups from PEMFG rats. Female rats belonging to the CG, SG and EMFG were sacrificed on postnatal day 32 by decapitation under deep anesthesia using an anesthetic drug (Ketalar® 50 mg/kg, Eczacıbaşı Co., Istanbul, Turkey). The animals' brains and cerebellums were then removed from the skulls, and the two organs were finally separated from each other.

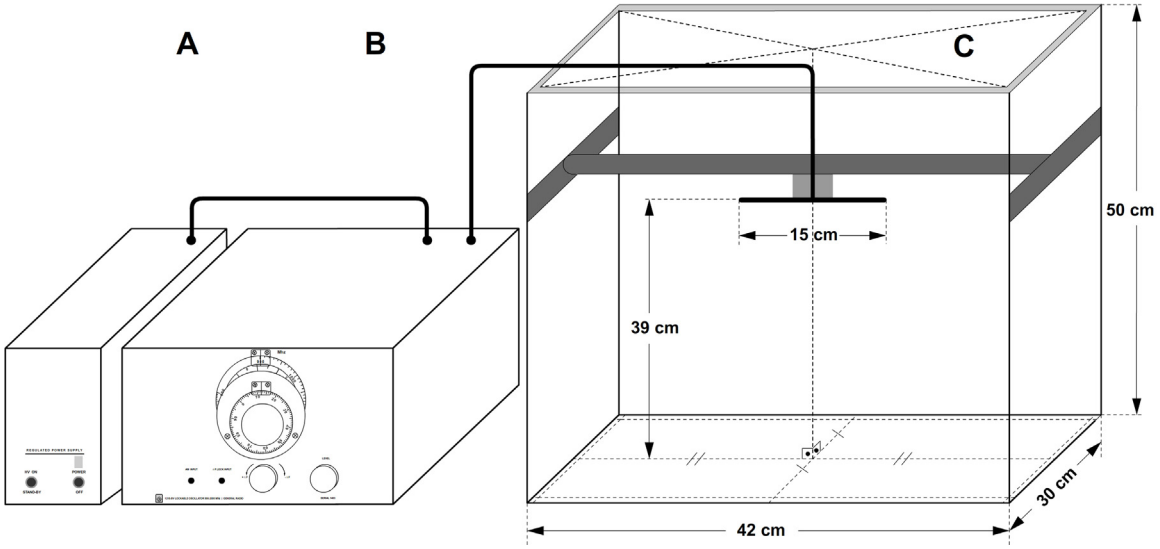

Fig. 1. Schematic representation of the EMF exposure system and the dimensions of the Plexiglas cage used to expose rats to 900 MHz EMF. (A) Power source. (B) Oscillator. (C) Plexiglas cage.

2.2. Histopathological and stereological procedures

After the cerebellums had been weighed on a sensitive device, they were fixed in formalin (10 days) for histopathological and stereological analyses. They were then routinely processed through graded alcohols and xylene solutions and embedded in melted granule paraffin to obtain fixed cerebellums in paraffin blocks. Serial sections 30 μm in thickness in the coronal plane were taken from the cerebellums in paraffin blocks using a fully automatic microtome (Leica RM 2255, Leica Instruments, Nussloch, Germany) with disposable metal microtome blades (Type N35, Feather Company, Osaka, Japan). Once the 30-μm-thick paraffin sections had been obtained, they were sampled according to the principles of the optical dissector counting method and systematic random sampling (Bas et al., 2009a, 2009b; Kaplan et al., 2012a, 2012b; West et al., 1991). Each sampled section of cerebellum was placed on a slide coated with a gelatin-formaldehyde mixture and stained with Cresyl violet dye. Histopathological observation and stereological analyses were then carried out on the Cresyl violet stained sections. A light microscope (Olympus, BX51, Japan) was used for histopathological examination, and micrographs were obtained using an Olympus DP 71 (Japan) camera microscope.

Purkinje cell numbers in the cerebellums of the male pups from the study groups were calculated using the optical dissector technique as described elsewhere (Gundersen and Jensen, 1987; Hausdorf et al., 2008; Kaplan et al., 2012a, 2012b; Tunc et al., 2007). The optical dissector technique was applied to cerebellum sections using a stereological workstation controlled by stereology software and equipment. This consists of a computer (Pentium PC, DELL OptiPlex, USA) containing stereology analysis software (Stereo Investigator 9, Computer Assisted Stereological Toolbox-Leica) designed for stereological research by MicroBright-Field (USA), a research microscope (Leica, DM4000B-M, Germany), a CCD camera (JVC, Japan), a measuring rod measuring on the Z axis (Heidenhain LIP401 R, Germany), a microcator indicator (Heidenhain ND 221 B, Germany), a computer-controlled step motor whose movements along the microscope table *X* and *Y* axes are controlled by PC and a tray joystick (Prior, USA). Purkinje cell counting was performed blind by one of the co-authors in order to obtain unbiased results using this stereological workstation.

At the beginning of the research, we performed a pilot study to define the sampling strategy and counting schedule. For this purpose, the section series to be analyzed was first systematically and randomly selected. Next, every successive 7th section was taken from the section series, giving a 1/7 section-sampling fraction. Approximately 12–18 sections from each cerebellum are known to be sufficient for estimating total neuron numbers for rodents (i.e. rats) in the optical dissector method (Odaci et al., 2004; Sonmez et al., 2010). Once the first section in the series had been selected, section sampling commenced from a random point (between 1 and 7). The efficiency of the sampling strategy and the number of sampled cells for total neuron number estimation have been corroborated using coefficient of error (CE) and coefficient of variation (CV) in previous stereological research (Bas et al., 2009b; Gundersen and Jensen, 1987; Odaci et al., 2010; West et al., 1991). Sampling reliability and the adequate number of sampled cells for calculating total neuron numbers were thus validated using CE and CV (Gundersen and Jensen, 1987). As reported previously, the CE should be ≤10% (Gundersen and Jensen, 1987; West et al., 1991). It was also possible to estimate the CV in the cerebellum in each group. These are valuable data for determining whether the number of subjects in each group is sufficient. The counting procedures employed, mean CV for each group, mean CE for stereological estimation of Purkinje cell numbers and other stereological parameters are presented in Table 1. After the study strategies obtained from the pilot study by means of CE and CV had been confirmed, Purkinje cell numbers in the cerebellums of the study groups were calculated on the basis of our pilot study strategies. Purkinje cells were counted if the largest neurons' nuclear profile came into focus within the unbiased virtual counting frames spaced randomly and systematically throughout the delineated regions (Bas et al., 2009b).

Total Purkinje cell numbers were determined on the basis of the number of Purkinje cells counted and the sampling probability (Bas et al., 2009b; West et al., 1991) (Table 1).

2.3. Statistical analysis

All computational analysis was performed on SPSS software (Statistical Package for the Social Sciences, version 11.0, SSPS Inc., Chicago, IL, USA). Significance was set at $p < 0.05$ for all tests. Values among the groups (CG, SG and EMFG) were analyzed using one-way ANOVA based on the results of the Levene and Shapiro Wilk tests for equality of variances and normality assumption, respectively ($p > 0.05$). Tukey's HSD multiple comparison test was then applied to determine any further differences among the groups.

3. Results

3.1. Histopathological observations

Histopathological evaluation of sections from CG, SG and EMFG rats was performed under light microscopy at various magnifications (Fig. 2). The CG (Fig. 2A1–A3) and SG (Fig. 2B1–B3) sections exhibited normal cerebellar morphology at all magnifications. No pathological change was observed in the cytoplasm of Purkinje or other cerebellar cells. However, some pathological changes such as pyknotic cells and neurons with dark cytoplasm were observed in EMFG sections during light microscopy at all magnifications (Fig. 2C1–C3). At high magnifications in particular we observed a disrupted typical Purkinje cell array. In addition, Purkinje cells were sparser in EMFG sections compared to both CG and SG.

3.2. Numbers of the Purkinje cell

Total Purkinje cell numbers obtained from stereological analysis were significantly lower in EMFG than in CG and SG ($p < 0.05$) (Table 1) (Fig. 3).

3.3. Physical examination and cerebellum weights

Physical examination of female rats from CG, SG and EMFG revealed no unexpected findings. No statistically significant difference was determined between the groups in terms of brain weights. There was also no significant difference between cerebellum weights in EMFG, CG and SG ($p > 0.05$) (Table 1).

4. Discussion

The prenatal effects of EMF emitted by different sources on human NS development are a subject of major concern (Gultekin and Moeller, 2013) since experimental studies have reported that exposure to EMF during gestational periods can result in

Table 1
Cerebellum weights, total Purkinje cell numbers and optical dissector analysis data for estimation of total Purkinje cell numbers in the cerebellums of CG, SG and EMFG rats.

Parameters	CG	SG	EMFG
Total Purkinje cell number (mean ± SD)	307,539 ± 10,818	308,548 ± 14,472	253,266 ± 11,066[a]
Cerebellum weight (mean ± SD)	0.195 ± 0.015	0.19 ± 0.012	0.197 ± 0.018
Dissector particle number (mean)	227	229	184
Number of sampled sections (mean)	18.1	17.3	17.8
Average section thickness (mean) (μm)	20.7	21.2	20.9
Number of steps for counting (mean)	153	158	129
Section sample (coronal)	1/7	1/7	1/7
CE (mean)	0.06	0.07	0.06
CV	0.03	0.04	0.03
Section-sampling fraction (ssf) (coronal)	1/7	1/7	1/7
Counting frame size (μm^2)	593	593	593
Area sampling fraction (asf) ($\mu m^2/\mu m^2$)	593/90.000	593/90.000	593/90.000
Thickness sampling fraction (tsf) (μm/μm)	10/20.7	10/21.2	10/20.9

$n = 6$ for each group; SD, standard deviation.
[a] EMFG Purkinje cell numbers significantly lower in comparison with both CG and SG ($p < 0.05$).

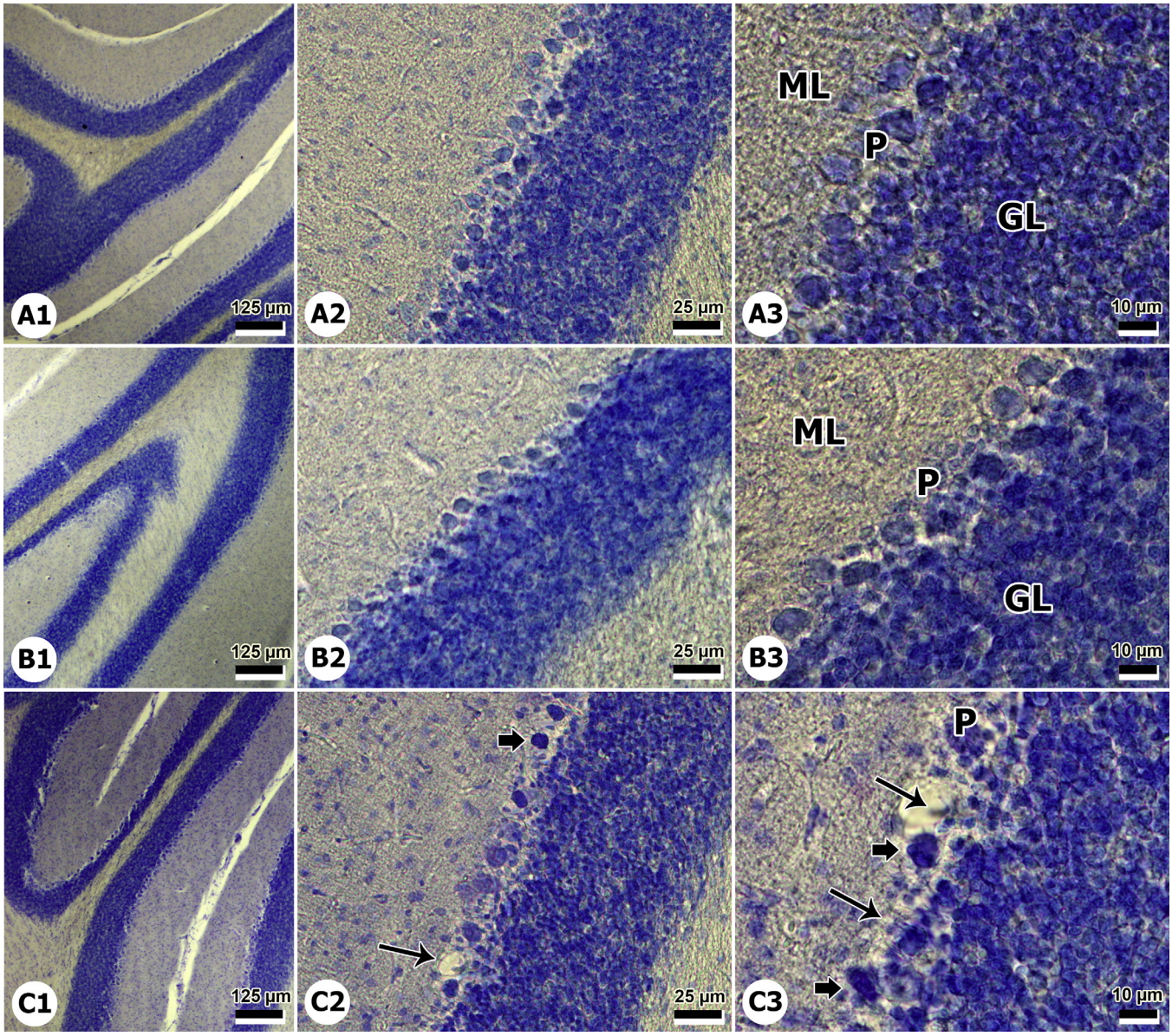

Fig. 2. Photomicrographs of female rat offspring cerebellum sections from CG (A1–A3), SG (B1–B3) and EMFG (C1–C3) are shown. Normal cerebellum morphology and neuron structure appear at different magnifications, with no pathological findings, in CG and SG, while substantial cell loss, disrupted Purkinje cell array (arrow), pyknotic neurons and neurons with dark cytoplasm (thick arrow) can be seen in EMFG. ML, molecular layer; GL, granular layer; P, Purkinje cell layer.

pathological effects on the development and functions of the NS (Eberhardt et al., 2008; Salford et al., 2003; Volkow et al., 2011). The effects of mobile phones on humans are of particular interest since these devices are generally held very near the brain (Corle et al., 2012; Davis et al., 2013; Inskip et al., 2010). In this study we used 900 MHz EMF since GSM-900 is a widely used cellular communication system in Europe and the most popular standard for mobile phones worldwide (Hancı et al., 2013; Odacı et al., 2015; Odacı and Özyılmaz, 2015; Topal et al., 2015; Türedi et al., 2014).

Prenatal and postnatal exposure to 900 MHz EMF has been reported to potentially cause various disturbances in the physiology, morphology and behavior of many animal species (İkinci et al., 2013; Odacı et al., 2013). For example, one study reported lower numbers of experimental group female rat pup cornu ammonis pyramidal cells following exposure to 900 MHz EMF during prenatal days 13–21. Pyknotic cells have also been identified in the cornu ammonis of experimental pups (Baş et al., 2013). Aldad et al. (2012) presented some evidence of neuropathology associated with cellular telephone radiation. They used a mouse model to demonstrate the effects of in utero EMF exposure from cellular telephones. The authors reported hyperactivity and memory compromise in mice exposed in utero using the object recognition, light/dark box and step-down assays. The authors concluded that these behavioral changes were due to altered neuronal developmental programming.

Haghani et al. (2013) investigated the potential effects of prenatal EMF exposure on the cerebellum of Wistar rat offspring. They exposed the EMF group rats to 900 MHz pulse-EMF for 6 h per day during the entire gestation period and reported that prenatal EMF exposure led to electrophysiological changes in Purkinje cells. However, these changes were not severe enough to affect cerebellum-dependent functional tasks. Recently, Razavinasab et al. (2014) reported behavioral, histological, and electrophysiological alterations in hippocampal function in Wistar rat offspring chronically subjected to mobile phone exposure during gestation. They exposed their mobile phone groups to 900 MHz pulsed-EMF irradiation for 6 h/day. Decreased neuronal excitability was detected in hippocampal pyramidal cells in the mobile phone

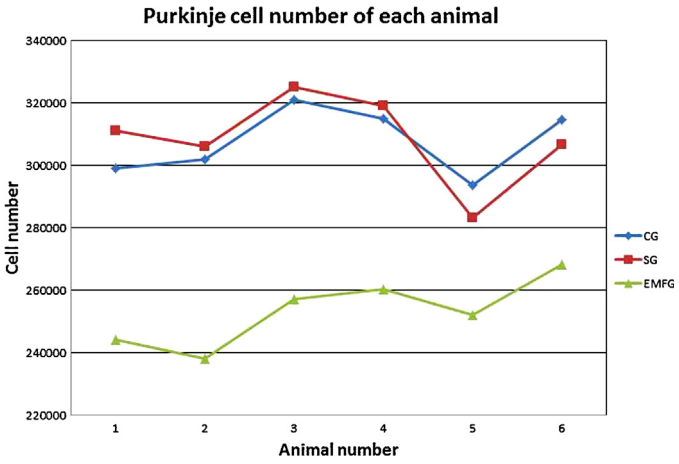

Fig. 3. The results of each female rat offspring' cerebellar Purkinje cell numbers were marked on the graph. SG and CG Purkinje cell numbers values approach one another as the number of animals rises; EMFG values, however, increasingly diverge from both SG and CG.

groups in both males and females. The authors concluded that that exposure to mobile phones adversely compromises cognitive functioning in female and male offspring rats, as determined using behavioral and electrophysiological techniques. Light microscopic examination of brain sections from control rats and those exposed to mobile phones revealed normal morphological architecture.

In our study we observed a number of histopathological changes, such as neurons with dark cytoplasm, pyknotic cells and a disrupted typical Purkinje cell array in EMFG sections. In addition, EMFG Purkinje cells were arranged quite sparsely compared to both CG and SG. The histopathological results of our research are supported by other studies. For example, Salford et al. (2003) identified neuronal injury to the cortex, hippocampus and basal ganglia of rats exposed for 2 h to EMFs of different wavelengths emitted by GSF mobile phones. That study reported scattered or clustered dark neurons, commonly shrunken and dark stained, homogenized with loss of discernible internal cell structures. It also reported an increase in dark neurons in the brains of GSM rats after GSM mobile phone EMF exposure. Another study investigated the effects of radiation from microwave ovens on pregnant and newborn rat brains. The most striking changes were observed in the non-ionizing radiation offspring group (exposed to non-ionizing radiation for 30 min), such as chronic inflammatory cell infiltrate, hemorrhage, necrotic neural tissues, and degenerative neurons with reactive glial proliferation (Inalöz et al., 1997).

In agreement with our own results, some studies have shown that prenatal or postnatal exposure to varying doses of EMF may result in structural damage and numerical decreases in Sprague Dawley rat offspring cerebellar neurons, such as Purkinje cells. Albert et al. (1981) combined three experiments in their study examining the effects of prenatal, postnatal, and pre- and postnatal-EMF exposure on Purkinje cells in the Sprague Dawley rat cerebellum. In the prenatal EMF exposure experiment, the pregnant rats were exposed daily from days 17 to 21 of gestation to 2450-MHz for 21 h/day. The offspring were investigated at 40 days post-exposure. A 26% decrease in Purkinje cell concentrations was detected in the cerebellums of prenatally exposed rats. In the pre- and postnatal-EMF exposure experiment, pregnant rats were exposed during days 16–21 days of gestation. The offspring of those pregnant rats were then exposed to continuous-wave 100-MHz EMF for 4 h/day over a 90-day period. Cerebellar morphology was investigated at 14 months post-exposure, and a 13% decrease in Purkinje cell concentrations was observed in the exposed rats. The authors reported that the changes observed in the pre- and perinatally exposed rats were probably irreversible, since the subjects were investigated one month after exposure. In the postnatal EMF exposure experiment, 6-day-old rat pups were exposed 7 h/day for 5 days to 2450-MHz EMF, and their cerebellums were studied either immediately or at 40 days after exposure. Twenty-five percent decreases were observed in Purkinje cell concentrations in the cerebellums of rats studied immediately after exposure, while there was no significant effect in the cerebellum at 40 days post-exposure. The authors suggested that the postnatal exposure effect might be reversible. Additionally, in the light of the results of the three experiments evaluated together, these suggested that EMF may affect the proliferation and migration process of Purkinje cells during Sprague Dawley cerebellar development.

In another study, 1- and 6-day old rat pups were exposed to continuous-wave 2450-MHz EMF for 5 days (7 h/day). Pups' cerebellar morphology was examined 1 day post-exposure. The results showed that postnatal exposure increased normal cell death, and the number of pyknotic cells determined in the experimental animals was almost double that observed in the controls. The authors also suggested that these effects might be reversible (Albert and Sherif, 1988). We observed a decrease in Purkinje cell numbers in the cerebellums of pups prenatally exposed to EMF and studied at postnatal day 32. However, there are some differences between our research and that study, such as variations in exposure duration and dosage. These differences resulted in the study results being interpreted differently. For example, Albert et al. (1981) and Albert and Sherif (1988) suggested that the effects observed may be reversible. We make no such claim, however. Another reason for the variation in interpretations of results may be the method used for cell calculation. We used the dissector method, a stereological technique, in the calculation of cell numbers. This is an unbiased and efficient morphometric method used to calculate numbers of countable arbitrary particles, such as neurons, under light microscope in a virtual 3D volume generated by two consecutive optical sections separated by distance t (Kaplan et al., 2012a, 2012b; Sterio, 1984). In addition to a significant decrease in Purkinje cell numbers in the cerebellum of EMFG compared to CG, we also observed a significance decrease in EMFG compared to SG. There were no pathological findings at physical examination and no statistical differences were observed in terms of cerebellum weight in EMFG, CG or SG.

In conclusion, our study results show that prenatal exposure to EMF affects the development of Purkinje cells in the female rat cerebellum and that the results of this pathological affect persist after the postnatal period. In other words, prenatal exposure to EMF may affect the proliferative activity and the process of migration of rat Purkinje cells during cerebellar development, since the growth and synaptic maturation of the rat Purkinje cells begin in the prenatal period and continue during the postnatal period (Altman, 1972). Therefore, since the neonatal period of NS development in rats is equivalent to the third trimester of NS development in humans (Dobbing, 1970; Dobbing and Sands, 1973; Jacobson, 1991; Rodier, 1980), it is possible that exposing humans to EMF during the critical period of NS development may result in changes in growth and synaptic maturation of Purkinje cells. However, further experiments are needed involving humans or non-human primates to determine the risk of EMF exposure during pregnancy.

Conflict of interest statement

There is no conflict of interest including any financial, personal or other relationships with other people or organizations that

could inappropriately influence, or be perceived to influence this work.

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